Lung Adenocarcinoma Harboring Mutations in the ERBB2 Kinase Domain

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Mutations in the *ERBB2* kinase domain have been reported in non-small cell lung cancer (NSCLC). Here, we describe a detailed search for *ERBB2* gene mutations in tumors derived from NSCLC patients. Tumor specimens from 223 patients who underwent resection for NSCLC were examined for the presence of mutations in exons 19 and 20 of the *ERBB2* gene. Correlations were then made between the expression of these mutations and the clinical characteristics of the patients from which they were derived as well as the tumor's pathological features. *ERBB2* mutations were observed in four of the above tumors (1.8%), all of which were adenocarcinomas. All *ERBB2* mutations were in-frame insertions that occurred in exon 20. The patients from whom these tumors were derived were nonsmokers. Three of the tumors were of the papillary subtype, and one was a mixed subtype that consisted of acinar, papillary, and solid components. None of the tumors had a bronchio-alveolar component nor did they have *epidermal growth factor receptor* or *K-ras* codon 12 mutations. In conclusion, patients with these tumors tended to be nonsmokers who had clinical features similar to those of lung cancer patients whose tumors expressed *epidermal growth factor receptor* mutations, although their tumors showed slightly different pathological features. (J Mol Diagn 2006, 8:351–356; DOI: 10.2353/jmoldx.2006.050132)

Members of c-erb B family of oncogenes, which include *epidermal growth factor receptor* (*EGFR*) and *ERBB2*, play a critical role in the development and progression of non-small cell lung cancer (NSCLC) by promoting cell growth and by preventing apoptosis by regulating downstream effectors such as mitogen-activated protein kinase, protein kinase B, and signal transducer and activator of transcription 3.^{1,2}

In 2004, Lynch and colleagues,³ Paez and colleagues,⁴ and Pao and colleagues⁵ reported that many NSCLC tumors, obtained from patients who responded to gefitinib or erlotinib treatment, harbored somatic mutations in the tyrosine kinase domain of their *EGFR* gene.

Several descriptive studies showed that *EGFR* gene mutations in NSCLC tumors were common in the lung adenocarcinomas of patients who were nonsmokers or mild smokers, women, and/or of Asian descent.^{6–13} These data suggest that *EGFR* mutations may play a causal role in the development of lung adenocarcinoma that does not correlate with smoking.

Stephens and colleagues¹⁴ first reported the presence of mutations in the kinase domain of the *ERBB2* gene, which shares a strong homology with the *EGFR* gene, in lung adenocarcinomas; Shigematsu and colleagues¹⁵ and Sasaki and colleagues¹⁶ reported similar mutations in their lung adenocarcinoma patients. Recently, Lee and colleagues¹⁷ also reported a similar mutation in a lung squamous cell carcinoma. The regions within which the *ERBB2* gene mutations occurred, ie, exons 19 and 20, correspond to the kinase domain of *EGFR* gene. Thus, these mutations could potentially result in the activation of the tyrosine kinase activity of the *ERBB2* protein and, as such, may play an important role in oncogenesis in a manner similar to *EGFR* mutations. In this study, we conducted a detailed search for *ERBB2* gene mutations in tumors derived from NSCLC patients who underwent tumor resection at a particular Japanese hospital.

Materials and Methods

Patients

A total of 223 patients with NSCLC who underwent tumor resection at the Department of Thoracic Surgery, Kyoto University Hospital from November 2002 to May 2005 were included in this study (Table 1). Included in this population were 154 patients whose tumors we previously examined for the presence of *EGFR* gene mutations.¹³ The patients' clinical data were obtained from in-patient and out-patient medical records and included chest X-ray films, whole body computed tomography films, bone scanning data, and operation records. Patients were classified as being either nonsmokers (who never smoked in a lifetime), former smokers (who stopped smoking at least 6 months before the time of diagnosis of lung cancer), or current smokers. Two hundred and five patients underwent complete lung, lobe, or

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Table 1. Characteristic Features of the 223 Patients that Participate in This Study

Characteristics	n	(%)
Age (years)		
Median	67	
Range	31 to 88	
Sex (no.)		
Men	137	(61.4)
Women	86	(38.6)
Smoking status (no.)		
Nonsmoker	79	(35.4)
Smoker	144	(64.6)
Former	35	(15.7)
Current	109	(48.9)
Tumor histology (no.)		
Adenocarcinoma	153	(68.6)
Squamous cell	52	(23.3)
Large cell	11	(4.9)
Other	7	(3.1)
Operation mode (no.)		
Pneumectomy (right)	1	(0.4)
Bilobectomy	1	(0.4)
Lobectomy	166	(74.4)
Segmentectomy	37	(16.6)
Partial resection	18	(8.1)
Pathological stage (no.)		
IA	87	(39.0)
IB	57	(25.6)
IIA	7	(3.1)
IIB	18	(8.1)
IIIA	35	(15.7)
IIIB	15	(6.7)
IV	4	(1.8)

segment removal as well as hilar and mediastinal lymph node dissection. Eighteen patients underwent partial lung resection and lymph node sampling. No patients were, at any time, exposed to gefitinib, erlotinib, or trastuzumab before their tumor was resected. Pathological staging was determined using the current tumor-node-metastasis classification system.¹⁸ Pathological diagnosis was determined, using the World Health Organization (WHO) classification system, by two pathologists of Kyoto University Hospital Laboratory of Anatomical Pathology who were unaware of the tumors' status, vis-à-vis the presence of mutations, and was confirmed by a third pathologist (T.M.).¹⁹ Many of adenocarcinomas were classified into mixed subtype according to the WHO classification system. Thus, to clarify the impact of *ERBB2* gene mutations on subtypes of adenocarcinoma, classification according to presence or absence of each component [bronchiolo-alveolar (BAC), papillary, acinar, and solid carcinoma with mucin (solid)] in a tumor was also used in this study. Written informed consent to perform genetic analyses was obtained from all patients before their surgery, and the study itself was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine.

Mutation Detection and Nucleotide Sequence Analysis

The method used to collect tumor samples was as we previously described.¹³ Since *ERBB2* mutations that

were recently reported in lung cancer specimens were located in exons 19 and 20,^{14–17} polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was used to screen for mutations in exons 19 and 20 of the *ERBB2* gene. PCR amplification was performed using HotStarTaq master mix (Qiagen K.K., Tokyo, Japan). The primers used for exon 19 were forward 5'-CACTCATATCCTCCTCTTTCTG-3' and reverse 5'-TGTCCTCCTAGCAGGAGAG-3', amplifying the 160-bp fragment; and for exon 20 were forward 5'-CCATACCCTCTCAGCGTAC-3' and reverse 5'-CGGAGAGACCTGCAAAGAG-3', amplifying the 249-bp fragment. The PCR was initially run at 95°C for 15 minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, and a final extension was run at 72°C for 10 minutes. SSCP analyses were performed using the GenePhor System and GeneGel Excel 12.5/24 (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions; the gel temperature was maintained at 15°C. To measure the sensitivity, we performed SSCP analysis of samples with a ratio of *ERBB2*-mutated copy [2322ins/dup(GCATACGTGATG)] to *ERBB2*-normal copy of 0.5, 0.2, 0.1, 0.05, 0.02, or 0.01, and we found that SSCP method was able to detect the mutation from the sample with a ratio of *ERBB2*-mutated copy to *ERBB2*-normal copy of 0.02 (data not shown). To determine the gel temperature for adequate optimization of altered band, we performed SSCP analyses under 12°C, 15°C, or 18°C of gel temperature, and we found that 15°C of gel temperature was most appropriate (data not shown). After the gels were stained with silver carbonate, altered bands were cut from the gels, and DNA fragments were eluted for direct sequencing. When an *ERBB2* mutation was observed, a DNA sample from the corresponding normal lung tissue was also analyzed for the presence of *ERBB2* mutations. We previously described the methods used for the detection of mutations in the *EGFR* gene, *p53* gene, and *K-ras* codon 12.¹³

Results

ERBB2 Gene Mutations in NSCLC

ERBB2 gene mutations were found in four of our patients (4 of 223, 1.8%); these mutations only occurred within exon 20. PCR-SSCP analysis revealed two types of altered bands in exon 20 (Figure 1), and nucleotide sequencing confirmed the presence of the following corresponding in-frame insertion mutations: 2322 ins/dup(GCATACGTGATG) was observed in three tumors, and 2326ins(TGT) with 2326G>T was observed in one tumor (Table 2). *ERBB2* mutations were exclusively observed in adenocarcinomas; thus, the incidence of *ERBB2* mutations in our adenocarcinoma patients was 2.6% (4 of 153). Corresponding normal lung tissues had no *ERBB2* mutations.

Figure 1. Single-strand conformational polymorphism in exon 20 of the *ERBB2* gene. W, Exon 20 wild-type control; patient 1-T, 2322 ins/dup(GCATACGTGATG); patient 1-NL, corresponding normal lung of patient 1; patient 2-T, 2326G>T, 2326ins(TGT); patient 2-NL, corresponding normal lung of patient 2. **Arrowheads**, altered bands.

Other Genetic Alterations

Mutations in the kinase domain of the *EGFR* gene (from exon 18 to exon 21) were found in 80 patients (80 of 223, 35.9%); all of these patients had an adenocarcinoma (80 of 153, 52.3%). Mutations within codon 12 of the *K-ras* gene were observed in the following 18 patients (8.1%): 15 adenocarcinoma patients (15 of 153, 9.8%), two squamous cell carcinoma patients (2 of 52, 3.8%), and one large cell carcinoma patient (1 of 11, 9.1%). Mutation types were as follows: substitution of cysteine (TGT) ($n = 7$), valine (GTT) ($n = 5$), aspartic acid (GAT) ($n = 4$), serine (AGT) ($n = 1$), and phenylalanine (TTT) ($n = 1$) in the place of glycine (GGT).

Seventy-one mutations within exons 5, 6, 7, and 8 of the *p53* gene were observed in the following 69 patients (69 of 223, 30.9%): 43 adenocarcinoma patients (43 of 153, 30.7%), 22 squamous cell carcinoma patients (22 of 52, 42.3%), and two large cell carcinoma patients (2 of 11, 18.2%), and in two patients with tumors of another histological type (two of seven, 28.6%). The frequency distribution of these mutations was 14 missense/nonsense point mutations and two insertions within exon 5; seven missense point mutations, three deletions, and three insertions within exon 6; nine missense point mutations, three deletions, and three insertions within exon 7; and 21 missense/nonsense point mutations and six deletions within exon 8.

Correlation Between the Presence of *ERBB2* Mutations and the Patients' Clinicopathological Features, as Well as Other Genetic Alterations

Because *ERBB2* mutations were observed exclusively in adenocarcinoma patients in our series, we investigated the relationship between the presence of *ERBB2* mutations and clinicopathological features/other genetic alterations in these adenocarcinoma patients ($n = 153$). Three of our four patients with *ERBB2*-mutated tumors were women, and all were nonsmokers (Table 3). Although statistical analyses could not be applied because of the low number of *ERBB2* mutations, clearly the incidence of *ERBB2* mutations was higher in female than male patients (3.8% versus 1.3%) and in nonsmokers than in smokers (5.3% versus 0%).

One of the above four tumors was staged as a grade 1 tumor, whereas the other three were staged as grade 2 (Table 2). Using the WHO classification system, three of these tumors were characterized as being of the papillary subtype, and one was characterized as a mixed subtype that consisted of acinar, papillary, and solid components. None of the tumors had a BAC component, which is in contrast to tumors with *EGFR* gene mutations, approximately half of which had a BAC component. Only one of our tumors had a solid component, and approximately half of those with a *K-ras* gene mutation had a solid component (Table 3).

EGFR mutations and *K-ras* codon 12 mutations were not detected in any of our *ERBB2*-mutated tumors. One of four *ERBB2*-mutated tumors had *p53* gene mutation [substitution of arginine (AGG) in the place of lysine (AAG) at the codon 164]. This type of point mutation (A:T to G:C) is not characteristic of mutations caused by tobacco smoking.²⁰ The presence of a *p53* mutation did not correlate with the presence of an *ERBB2* gene mutation (Table 4).

Discussion

ERBB2 gene mutations were detected in four of 223 patients (1.8%) who underwent resection for NSCLC; our results are comparable to those of previous reports (prevalence, 0.9 to 4.2%; Table 5).¹⁴⁻¹⁷ A 12-bp in-frame insertion mutation within exon 20, which was the major type of mutation found by other investigators,¹⁴⁻¹⁷ was

Table 2. Patients Harboring *ERBB2* Gene Mutations in Their Tumor

Age (years) Sex	Smoking	Tumor size/ TNM classification	Differentiation grade/ adenocarcinoma subtype	Nucleotide alteration	Amino acid alteration
45 Woman	Non	15 mm t1n0m0, IA	Well Papillary	2322 ins/dup (GCATACGTGATG)	ins774(AYVM)
65 Man	Non	22 mm t1n1m0, IIA	Moderate Mixed (acinar/papillary/solid)	2326G>T 2326ins(TGT)	G776L, ins776(C)
79 Woman	Non	8 mm t1n0m0, IA	Moderate Papillary	2322ins/dup (GCATACGTGATG)	ins774(AYVM)
82 Woman	Non	20 mm t2n0m0, IB	Moderate Papillary	2322 ins/dup (GCATACGTGATG)	ins774(AYVM)

Table 3. Correlation Between the Presence of *ERBB2* Mutations and the Patients' Clinicopathological Features

Variable	Mutated gene				
	Adenocarcinoma patients (n = 153)	<i>ERBB2</i> (n = 4)	<i>EGFR</i> (n = 80)	<i>K-ras</i> codon 12 (n = 15)	Not detected (n = 54)
Age (years)					
Median (range)	67 (31 to 83)	72 (45 to 82)	65 (33 to 83)	65.5 (50 to 79)	63 (31 to 83)
Gender (no.)					
Men	75	1	22	10	42
Women	78	3	58	5	12
Smoking history (no.)					
Nonsmoker	75	4	60	3	8
Former smoker	25	0	12	3	10
Current smoker	53	0	8	9	36
Tumor differentiation (no.)					
Well	42	1	28	3	10
Moderate	65	3	37	5	20
Poor	46	0	15	7	24
Subtypes of adenocarcinoma (no.)					
Mixed	115	1	61	11	42
Bronchio-alveolar	6	0	3	1	2
Papillary	26	3	15	1	7
Acinar	3	0	1	0	2
Solid	3	0	0	2	1
Acinar component (no.)					
Present	96	1	46	11	38
Absent	57	3	34	4	16
Bronchio-alveolar component (no.)					
Present	59	0	43	3	13
Absent	94	4	37	12	41
Papillary component (no.)					
Present	128	4	70	10	44
Absent	25	0	10	5	10
Solid component (no.)					
Present	48	1	15	8	24
Absent	105	3	65	7	30

the most frequent mutation in our patients. Other mutations that we found included a novel 2326G>T, 2326ins(TGT). In this type of mutation, the point in exon 20 at which the insertion mutation occurred corresponded to the point in exon 20 of the *EGFR* gene at which insertion mutations were found. This finding suggests that one or just a few compounds with DNA editing capabilities may be responsible for the development of mutations within the tyrosine kinase domain of the *ERBB2* and *EGFR* genes. Clarification of the mechanism by which these mutations occur would undoubtedly lead to advances in our understanding of oncogenesis and its prevention.

As with *EGFR* mutations, *ERBB2* mutations were more prevalent in female and nonsmoking patients although, again, our numbers were small. These findings are similar to those of Shigematsu and colleagues¹⁵ and Sasaki and colleagues¹⁶ but different from those of Stephens and colleagues¹⁴ and Lee and colleagues.¹⁷ Further investi-

gation will be needed to clarify the relationship between these variables although, at least in Japanese patients, it appears that the absence of a smoking history and being of the female sex are factors that are linked to the expression of *ERBB2* mutations.

All adenocarcinomas that harbored *ERBB2* mutations had a papillary component while none had a BAC component. This histological predilection is slightly different from that seen for *EGFR* mutations in adenocarcinomas, in which approximately half of the tumors displayed BAC features.¹³ It remains to be seen whether this discrepancy is real or merely a reflection of the low number of mutated tumors that were examined in our study; detailed histological examinations of this type were not performed in previously published studies.

K-ras and *EGFR* mutations were not observed in any of the *ERBB2*-mutated tumors in our patients, confirming previous reports.^{14,15,17} *ERBB2*, *EGFR*, and *K-ras* are

Table 4. Correlation Between the Presence of *ERBB2* Mutations and Other Genetic Alterations in 153 Adenocarcinoma Patients

Number of adenocarcinoma patients (%)	Mutation status			
	<i>ERBB2</i>	<i>EGFR</i>	<i>K-ras</i> codon 12	<i>p53</i> (mutated/wild)
4 (2.6%)	Mutated	Wild	Wild	1/3
80 (52.2%)	Wild	Mutated	Wild	17/63
15 (9.8%)	Wild	Wild	Mutated	4/11
54 (35.3%)	Wild	Wild	Wild	21/33

Table 5. Summary of Previous Reports on *ERBB2* Mutations in Lung Cancer

Reference	Frequency in		Patient ethnicity	Age/ gender	Smoking (pack-year)	Stage	Histology	Nucleotide alteration
	Overall	Adenocarcinoma						
Stephens et al ¹⁴	5/120 (4.2%)	5/51 (9.8%)	UK	ND	Unknown*	ND	Ad	2322ins/dup(GCATACGTGATG)
			UK	ND	Unknown*	ND	Ad	2322ins/dup(GCATACGTGATG)
			UK	ND	Unknown*	ND	Ad	2322ins/dup(GCATACGTGATG)
			UK	ND	Unknown*	ND	Ad	2335ins(CTGTGGGCT)
			UK	ND	Unknown*	ND	Ad	2263-2264TT>CC
Shigematsu et al ¹⁵	11/671 (1.6%)	11/394 (2.8%)	Japan	74 years/F	Non	I	Ad	2324ins/dup(ATACGTGATGGC)
			Japan	58 years/F	Non	I	Ad	2324ins/dup(ATACGTGATGGC)
			Japan	58 years/M	Non	I	Ad	2324ins/dup(ATACGTGATGGC)
			Japan	63 years/M	Smoker (40)	I	Ad	2324ins/dup(ATACGTGATGGC)
			Japan	58 years/F	Smoker (0.5)	I	Ad	2326ins(TTT)
			Japan	66 years/F	Non	III	Ad	2326ins(TTT), 2326G>C
			Japan	52 years/F	Smoker (5)	III	Ad	2239ins(GGGCTCCCC)
			Japan	68 years/F	Non	I	Ad	2240ins(GGCTCCCCA)
			Taiwan	76 years/F	Non	I	Ad	2324ins/dup(ATACGTGATGGC)
			Taiwan	72 years/M	Non	II	Ad	2326ins(TTT)
Sasaki et al ¹⁶	1/95 (1.1%)	1/71 (1.4%)	Australia	83 years/M	Non	I	Ad	2325ins/dup(TACGTGATGGCT)
			Japan	ND/F	Non	ND	Ad, well-differentiated	2324ins/dup(ATACGTGATGGC)
Lee et al ¹⁷	1/114 (0.9%)	Not analyzed	Korea	65 years/M	Smoker (60)	IB	Sq	2305G>C

Ad, adenocarcinoma; Sq, squamous cell carcinoma; ND, not described. *Unknown: in the study reported by Stephens et al,¹⁴ four of five patients were smokers, but further information was not described.

important molecules that are responsible for the regulation of the mitogen-activated protein kinase pathway.²¹⁻²³ *ERBB2* and *EGFR* also play a role in the regulation of the phosphatidyl inositol triphosphate kinase-Akt pathway.^{22,23} It is likely that *ERBB2*, *EGFR*, and *K-ras* codon 12 mutations have similar effects vis-à-vis the oncogenesis of lung adenocarcinomas. Unlike *K-ras* mutations that appear to be involved in the development of adenocarcinomas in smokers, *ERBB2* and *EGFR* gene mutations may play a key role in the development of adenocarcinomas in nonsmokers.

The above conclusions notwithstanding, our study had two limitations. Mutations outside of exons 19 and 20 were not examined, and the PCR-SSCP method could have overlooked mutations that did not appear as band pattern alterations. Thus, the incidence of *ERBB2* mutations that we detected may have been underestimated. However, our results on the incidence of *ERBB2* mutations seemed to support those of previous investigators, and condition settings in the SSCP analysis were stringently performed in this study. Therefore, we think our results have sufficient validity.

In conclusion, mutations in the *ERBB2* gene were found in 2.6% of our Japanese adenocarcinoma patients and relatively infrequent. *ERBB2*, *EGFR*, and *K-ras* codon 12 mutations were mutually exclusive. On the basis of four adenocarcinomas with *ERBB2* gene mutations in this series, *ERBB2* gene mutations tended to be found in nonsmokers and in adenocarcinomas that had a different histological pattern from those with *EGFR* gene mutations.

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